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APPLICATION

FOR

UNITED STATES LETTERS PATENT

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TITLE: FORMATION OF SUPERPARAMAGNETIC PARTICLES

FORMATION OF SUPERPARAMAGNETIC PARTICLES

Cross-Reference to Related Applications

This application is a continuation-in-part of Application No. 10/352,280 filed January 27, 2003, which is incorporated herein by reference.

Field of the Invention

The present invention relates to methods for the preparation of superparamagnetic iron particles. The superparamagnetic materials are formed under alkaline conditions by the oxidation of a ferrous ion-polysaccharide matrix with the mild oxidizing agent, nitrate. The present invention further relates to methods for preparing superparamagnetic iron compositions. These compositions are useful for the separation, isolation, identification, or purification of biological materials.

Background of the Invention

Magnetic particles are used for a variety of separation, purification, identification, or isolation techniques in connection with biological molecules. Typically, a magnetic particle is coupled to a molecule capable of interacting with another molecule or cell in a biological sample. This interaction can either be specific, e.g., the specific binding between an epitope and a binding region for that epitope, or general, e.g., hydrophobic or ionic interactions. Once a biological sample is brought into contact with the magnetic particle, those biological entities that bind to the magnetic particle are subsequently isolated by application of a magnetic field. Such magnetic separation techniques have been employed to sort cells, to recover antibodies or enzymes from a solution, to purify proteins using affinity techniques, or to remove unwanted particles from a suspension of

biological materials. (Ugelstad et al., "Monodisperse magnetic polymer particles. New biochemical and biomedical applications" *Blood Purif.*, 11(6):349-69 (1993); Setchell, "Magnetic separations in biotechnology – a review" *J. Chem. Tech. Biotechnol*, 35B:175-82 (1985))

An important feature of magnetic separation is the economy and physicochemical characteristics of the magnetic support. High mechanical resistance and resistance to solvent and microbial attack make inorganic magnetic materials excellent supports, but they lack in functional groups for selective binding of biomolecules of interest. Therefore, inorganic magnetic material is most commonly coated with polymers. Traditionally, the magnetizable particles used for bioseparations have been divided into four general classes:

1. Core-and-shell beads with a magnetic core and a hard shell coating of polymerized monomer or a silanizing agent, e.g., U.S. Patent No. 4,267,234 (polyglutaraldehyde shell around ferrofluid core particles), U.S. Patent No. 4,454,234 (suspension or emulsion polymerized coating around submicron magnetic particles), U.S. Patent Nos. 4,554,088, 4,695,392 and 4,695,393 (silanized magnetic oxide particles of polydisperse size and shape), U.S. Patent No. 4,672,040 (polysilane coated magnetic particles), U.S. Patent No. 4,783,336 (suspension polymerized polyacrolein around ferrofluid particles), U.S. Patent No. 4,795,698 (bovine serum albumin coating), and U.S. Patent No. 4,964,007 (gelatin-gum arabic-surfactant coating);

2. Core-and-shell beads with a magnetic core and a loose shell of random coil or globular polymer that may or may not be crosslinked, e.g., U.S. Patent No. 4,452,773 (dextran coating around ferrofluid particles) and U.S. Patent No. 4,795,698 (protein such as bovine serum albumin around ferrofluid particles;

3. Magnetic latex materials formed by uniformly embedding ferrofluid particles in polystyrene latex particles, e.g., U.S. Patent No. 4,358,388; and

4. Porous polymer particles filled with magnetic materials, such as polymer-ferrite or polymer maghemite composite systems, Nustad et al.,

"Monodisperse Polymer Particles In Immunoassays And Cell Separation", *Microspheres: Medical and Biological Applications*, A. Rembaum and Z. Tokes, Eds. (Boca Raton, Fla.: CRC Press, 1988) pages 53-75, C.D. Platsoucas et al., "The Use of Magnetic Monosized Polymer Particles For The Removal Of T Cells From Human Bone Marrow Cell Suspensions", *ibid.* at pages 89-99, and U.S. Patent Nos. 4,563,510, 4,530,956 and 4,654,267.

Magnetically responsive composite microparticles including magnetically responsive materials and porous solid water-insoluble matrices such as proteinaceous materials, polysaccharides and the like, have also been described, e.g., U.S. Patent No. 4,169,804.

In addition, magnetic cellulose fibers and paper may be prepared by synthesizing ferrites *in situ*, e.g., U.S. Patent No. 5,143,583. These fibers have been prepared via careful O_2 oxidation of ferrous hydroxide and precipitated with NaOH from the ferrous ion-exchanged form of the matrix. The chemistry yields magnetic fibers containing small superparamagnetic ferrite (Fe_3O_4) particles of about 10 nm in size. Typically, carboxymethylated cellulose fibers are used as the material subjected to the magnetization scheme, but it has also been suggested that the process could be practiced with a wide range of natural biopolymers.

Lianes et al., *Int. J. of Polymeric Mat.* 51:537-45 (2002), disclose superparamagnetic composites of alginate, starch, and chitosan for use in drug delivery or cell sorting, where Fe_2O_3 was formed inside these matrices by oxidation of Fe(II) with hydrogen peroxide.

Particulate ferromagnetic, ferrimagnetic and superparamagnetic agents have also been proposed for use as negative MR contrast agents. Examples of materials which may be used in this way as stabilizers include carbohydrates such as oligosaccharides and polysaccharides, as well as polyamino acids, oligonucleotides and polynucleotides and polyalkylene oxides (including poloxamers and poloxamines), and other materials as proposed in U.S. Patent Nos. 5,464,696 and 4,904,479.

Given the many uses of matrices of superparamagnetic particles that interact with biological materials and, particularly, those matrices that are polysaccharide-based, there remains a need in the art for a method of preparing superparamagnetic particles where Fe(II) is oxidized in the presence of a polysaccharide-based matrix under mild and efficient conditions. Polysaccharide matrices are particularly desirable for *in vitro* or *in vivo* use as they typically do interact non-specifically with proteins (e.g., they do not have attractive hydrophobic interactions with proteins). Ideally, the method of iron oxidation employed in a polysaccharide-based matrix to yield a superparamagnetic particle or composition should proceed in high yield with minimal or no oxidative degradation of the matrix. Additionally, it is desirable that the oxidation be performed *in situ*, so that the process is scalable and easy to control. It is also desirable that this method produces particles or compositions useful for the separation, isolation, identification, or purification of biological materials.

Summary of the Invention

In a first aspect, the invention features a method for the *in situ* formation of superparamagnetic particles in a polysaccharide matrix where an Fe(II) salt is diffused into a starch matrix, thereby entrapping Fe(II) ions within the matrix, and where the Fe(II) ions are oxidized with nitrate under alkaline conditions, converting the starch matrix of Fe(II) ions into superparamagnetic ferric oxide particles.

In an embodiment of the first aspect, the polysaccharide matrix can be starch, cross-linked starch, chitosan, chitin crystallites, dextran, cross-linked dextran, cellulose, cellulose fibers, microcrystalline cellulose, alginic acid, hyaluronic acid, glycogen, or a glycosylaminoglycan. Desirably, the polysaccharide matrix can be cross-linked starch, chitosan, chitin crystallites, cross-linked dextran, cellulose fibers, or microcrystalline cellulose, and most desirably, the polysaccharide matrix can be cross-linked starch or chitosan.

In another desirable embodiment of the invention, alkaline conditions are provided by contacting the matrix with ammonium hydroxide. Additional embodiments include the use of sodium nitrate, potassium nitrate, cesium nitrate, ammonium nitrate, tetra(C_1-C_8 alkyl)ammonium nitrate, or barium nitrate as the oxidant, with the most desirable oxidant being sodium nitrate. It is also most desirable to use $FeCl_2$ as the source of Fe(II) ions.

In a second aspect, the invention features a method for the *in situ* oxidation of a superparamagnetic particle polysaccharide matrix which includes the steps of, a) maintaining a solution or a suspension of said superparamagnetic iron particle at a pH of about 7.5 to about 10.5 by the addition of a base, b) maintaining the basic solution/suspension of said superparamagnetic iron particle at a temperature of about 0 °C to about 20 °C, c) adding sodium bromide and a catalytic amount of TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy, free radical) to the solution/suspension, and d) adding sodium hypochlorite to the solution/suspension. As an embodiment of the second aspect, sodium chlorite may be added as an additional oxidant after the addition of TEMPO to the reaction mixture. In an additional embodiment, the superparamagnetic particle that is oxidized is one that is prepared by the method of the first aspect of the invention.

In a third aspect, the invention features a composition prepared by the method of the second aspect of the invention, having a starch-based matrix and a superparamagnetic iron oxide particle within this matrix, wherein the matrix is about 5 percent to about 30 mole percent carboxyl groups.

In a fourth aspect, the invention features a composition formed using the method of the first or second aspect, wherein the composition includes a polysaccharide matrix and a superparamagnetic iron oxide particle within the polysaccharide matrix. This composition may also include a second biological molecule different than the polysaccharide included in the matrix. Desirably, the second biological molecule is covalently attached to the polysaccharide matrix.

In a fifth aspect, the invention features a method for the separation, isolation, identification, or purification of a biological material using a composition of the invention that includes contacting the composition with the biological material and affecting the separation, isolation, identification, or purification with a magnetic field. In an embodiment of this aspect, the composition includes a biological molecule that interacts positively with the biological material. The biological material can be a cell, a virus, or a phage. Desirably, the biological material is a protein, a peptide, a carbohydrate, a glycopeptide, a glycoprotein, a glycosylaminoglycan, a cationic lipid, a glycolipid, or a polynucleotide. Most desirably, the biological material is a protein.

In a sixth aspect, the invention features a method of separating a target biological material from a mixture. The method includes the step of contacting the mixture with a superparamagnetic polysaccharide matrix in an aqueous solution having a pH of between 3 and 10, wherein the superparamagentic polysaccharide matrix is prepared by a method including the steps of a) diffusing an Fe(II) salt into the polysaccharide matrix, thereby entrapping Fe(II) ions within the matrix; and b) oxidizing the entrapped Fe(II) ions with nitrate under alkaline conditions to convert the Fe(II) ions into superparamagnetic ferric oxide particles.

In an embodiment of the sixth aspect, the polysaccharide matrix can be starch, cross-linked starch, chitosan, chitin crystallites, dextran, cross-linked dextran, cellulose, cellulose fibers, microcrystalline cellulose, alginic acid, hyaluronic acid, glycogen, or a glycosylaminoglycan. Desirably, the polysaccharide matrix can be cross-linked starch, chitosan, chitin crystallites, cross-linked dextran, cellulose fibers, or microcrystalline cellulose, and most desirably, the polysaccharide matrix can be cross-linked starch or chitosan.

In yet another embodiment of the sixth aspect, the superparamagnetic polysaccharide matrix further includes a ligand having affinity for the target biological material. The ligand may be either entrapped within the matrix or covalently attached to the matrix. The ligand having affinity for the target

biological material can include a protein, a peptide, a carbohydrate, a glycopeptide, a glycosylaminoglycan, a cationic lipid, a glycolipid, or a polynucleotide. Exemplary affinity ligands include, without limitation, antibodies or antibody fragments, antigens, biotin, steptavidin, enzyme substrates or substrate analogs, protein A or protein A analogs, and complementary polynucleotides.

In an embodiment of the sixth aspect, the target biological material is selected from protozoa, bacteria, fungi, yeast, cultured cells from multicelled organisms, viruses, organelles, suborganelles, proteins, glycoproteins, vaccines, such as Comvax™ (Merck), Engerix-B (SmithKline Beecham), LYMErix™ (SmithKline Beecham), and RECOMBIVAX HB® (Merck), lipoproteins, carbohydrates, lipids, and fragments thereof.

Desirably, the target biological material is a protein. Proteins that can be separated using the methods described herein include albumins; angiogenic factors; antibodies, such as antibodies to immunoglobulins, e.g., IgA, IgD, IgE, IgG, and IgM classes, and isotypes thereof (e.g., IgG2 and IgG4), trastuzumab (Genentech), oprelvekin (Genetics Institute), muromonab-CD3 (Ortho Biotech), infliximab (Centocor), abciximab (Eli Lilly), ritiximab (Genentech), basiliximab (Novartis), palivizumab (MedImmune), thymocyte globulin (SangStat), cetuximab (ImClone), and daclizumab (Hoffman-La Roche); antihemophilic factor; bactericidal/permeability increasing protein rBPI-21 (e.g., Neuprex™, Baxter Hyland); calcitonin; ceredase; clotting factors, such as factor IV, factor VIII, factor IX, and factor VIIa; colony stimulating factors, such as GM-CSF and G-CSF; cytokines, such as TNF-alpha; interferons, such as interferon alpha, interferon beta, and interferon gamma; enzymes, such as RNase, DNase, proteases, urate oxidase, adenosine deaminase, alronidase, alpha galactosidase, and alpha glucosidase; growth factors, including vascular endothelial growth factor (VEGF), endothelial cell growth factor (ECGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and platelet derived growth factor (PDGF); growth hormones, such as human growth hormone and bovine growth hormone;

differentiation factors; fibrin; follicle-stimulating hormone (FSH); glucocerebrosidase; herudin; immune globulins, antithymocyte globulin, hepatitis B immune globulin, and CMV immune globulin; insulin; interleukins, such as interleukin-2 and interleukin-11; leptin; luteinizing hormone (LH); osteogenic protein-1; osteoprotegerin; platelet activating factor-acetylhydrolase (rPAF-AH); parathyroid hormone rhPTH (e.g., ALX1-11, NPS Allelix); poetins, such as erythropoietin and thrombopoietin; prolactin; relaxin; RSV; somatotropin-releasing hormones; tachykinins; thyroid-stimulating hormone (TSH); thyrotropin alfa; rhIGF-I/rhIGFBP-3 complex; LFA-3/IgG1 human fusion protein (e.g., Amevive™, Biogen); and tissue plasminogen activator.

In another embodiment of the sixth aspect, the contacting is performed in the presence of a buffer. Exemplary buffers that can be used in the methods of the invention include, without limitation, acetate, citrate, phosphate, tris(hydroxymethyl)amino methane (Tris), glycine, carbonate, lactate, pivalate, pyridine, picolinate, succinate, histidine, N-Morpholinosulfonic acid (Mes), Bis-(-2-hydroxyethyl)imino-tris-(hydroxymethyl)methane, (Bis-Tris), N-(2-acetamido)-2-aminoethane sulfonic acid (Aces), imidazole, N-morpholinopropane sulfonic acid (Mops), N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (Tes), triethanolamine, N-tris(hydroxymethyl)methyl-glycine (Tricine), tris(hydroxymethyl)aminopropane sulfonic acid (Taps), 2-amino-2-methyl-1,3-propanediol, diethanolamine, taurine, ammonia, borate, ethanolamine, aminopropan-3-ol, and combinations thereof.

In another embodiment of the sixth aspect, the target biological material is rinsed from the matrix using a solution of high conductivity. For example, the target protein can be efficiently removed by rinsing the matrix with a salt solution (e.g., having a salt concentration of 20 mM, 50 mM, 100 mM, 250 mM, 500 mM, 750 mM, 1 M, or greater), with a solution having a low pH (e.g., less than 5.0), or with a solution having a high pH (e.g., greater than 8.5).

In another embodiment of the sixth aspect, the mixture from which the target protein is separated includes between about 10 mg/L and 5 g/L or, more desirably, between about 500 mg/mL and 3 g/L target biological material. Typically, the mixture includes an unclarified fermentation broth or cell lysate or protein mixture. Alternatively, the mixture is clarified prior to contacting the matrix.

In still another embodiment of the sixth aspect, the method of separating the target biological material from the mixture includes the step of applying a magnetic field to the mixture after the step of contacting the mixture with a superparamagnetic polysaccharide matrix described herein.

In yet another embodiment of the sixth aspect, the contacting occurs while performing packed column chromatography or during expanded bed adsorption to separate a target biological material from a mixture.

In an embodiment of any of the above aspects, the polysaccharide matrix further includes charged groups. The charged groups can be selected, without limitation, from carboxyl, ammonium, sulfate, or combinations thereof. For example, the matrix can include between about 1 percent to about 30, 40, 50, 60, 70, 80, 100, 120, 140, 160, 180, or even 200 mole percent carboxyl groups per saccharide unit.

By the term "affinity ligand" is meant a moiety that binds selectively or preferentially to a component of the target material to be isolated, purified, separated, or analyzed through a specific interaction with a binding site of the component. In the practice of the present invention, the affinity ligand is typically associated with the superparamagnetic particle or composition prepared by the method of the invention. Examples of affinity ligands that may be useful in the method of the present invention include: protein A and protein A analogs, which selectively bind to immunoglobulins; dyes; antigens, useful for purification of associated antibodies; antibodies, for purification of antigens; substrates or

substrate analogs, for purification of enzymes; complementary polynucleotides; and the like.

In the present context, the term "alkaline conditions" refers to conditions where the pH of a solution is greater than neutrality, *i.e.*, pH > 7.0. These conditions can be achieved by the addition of an organic or inorganic base to an aqueous solution or to a mixed aqueous/organic solution.

By "biological material" is meant a substance that is naturally occurring, derived from a substance that is naturally occurring, or an analog of a substance that is naturally occurring. Biological entities include cells, viruses, phages, and the like. Biological entities also include biological molecules, as defined below.

By "biological molecule" is meant a substance that contains naturally occurring units, subunits, or analogues thereof. Biological molecules include, without limitation, proteins, peptides, carbohydrates, glycopeptides, glycoproteins, glycosylaminoglycans, cationic lipids, glycolipids, or polynucleotides. In addition, biological molecules may be synthetic molecules containing unnatural amino acids, unnatural nucleotides, and the like. Biological molecules may also be those entities derived from recombinant technology.

By "carbohydrate" is meant any group of organic compounds based on the general formula $C_x(H_2O)_y$, or a derivative thereof. Carbohydrates include monosaccharides, oligosaccharides, and polysaccharides. Carbohydrates may also vary from this general formula and include deoxy-compounds, such as 2-deoxy-D-ribose, where one or more hydroxy groups of the carbohydrate are replaced by hydrogen.

By "Contramid®" is meant the proprietary excipient of Labopharm, Inc., based on modified high amylose starch.

By "diffusing" is meant the dissemination of a substance in a matrix. Diffusing is usually accomplished by mixing or by waiting a sufficient time period after a substance comes into contact with a matrix material for the substance to be evenly distributed within the matrix.

By "entrapment" is meant the binding of a substance in a matrix. Binding may be either through covalent or non-covalent interactions.

By "glycosylaminoglycan" is meant a carbohydrate wherein one or more than one hydroxyl groups are replaced with amino groups or derivatized amino groups (e.g., N-acetyl groups).

The phrase "interacts positively" is meant to describe any contact between two entities, typically two different biological entities, which results in a thermodynamically favorable interaction. Typically, this interaction is between an affinity ligand and its biological cognate. Other examples of positive interactions are thermodynamically favorable hydrophobic interactions or thermodynamically favorable ionic interactions.

By "lipid" is meant any member of fatlike substances that occur in living organisms. The term lipid may include: fatty acids, triacylglycerols or other fatty acid esters, long chain alcohols and waxes, sphingoids or other long-chain bases, glycolipids, phospholipids, sphingolipids, carotenes, polyprenols, sterols, and terpenes. The term "cationic lipid" refers to a lipid with one or more positive charges. The term "glycolipid" refers to a lipid that is covalently bound to a carbohydrate.

By "MagCon" is meant high amylose crosslinked starch that has been converted to the superparamagnetic state.

By "MagConC" is meant MagCon that has been prepared from Contramid®.

By "MagCon-30COOH" is meant Contramid®, converted to the superparamagnetic state, which has also been modified to contain carboxyl groups. The mole percentage of carboxylation is designated by the number preceding COOH (e.g., MagCon-30COOH is 30% carboxylated). The degree of carboxylation can be from 0% up to and including 200% or two carboxyls per glucose unit. The percent carboxylation is determined by a comparison of the solid-state ^{13}C-NMR spectra of MagCon and the carboxylated derivative.

By "MagChi" is meant chitosan that has been converted to the superparamagnetic state.

By "MagChi-Protein A" is meant MagChi to which has been covalently bound Protein A.

By "MagCon-NH_2" is mean a MagCon particle to which chitosan has been grafted to the surface (e.g., chemically coupled using chemistry described herein).

By "MagMCC" is meant microcrystalline cellulose (e.g. Avicel®) that has been converted to the superparamagnetic state.

By "matrix" is meant a binding substance or material. Binding may be either through covalent or non-covalent interactions. In general, the matrices of the invention are polymeric, or mixtures of polymeric functionalized organic compounds. Exemplary matrices are starch, chitosan, or other polysaccharides.

By "monosaccharides" are meant polyhydric alcohols from three to ten or more carbon atoms containing either and aldehyde group (e.g., aldoses) or a keto group (e.g., ketoses), or masked aldehyde or keto groups, or derivatives thereof. Examples of monosaccharide units are the D and L configurations of glyceraldehyde, erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, talose, dihydroxyacetone, erythrulose, ribulose, xylulose, puscose, fructose, sorbose and/or tagatose. Examples of monosaccharides also include those monosaccharide deoxy sugars, such as, for example, fucose, rhamnose, and digitoxose; deoxyamino sugars such as, for example, glucosamine, mannosamine, galactosamine; deoxyacylamino sugars such as, for example, N-acetylglucosamine, N-acetylmannosamine, and N-acetylgalactosamine; and aldonic, aldaric and/or uronic acids such as, for example, gluconic acid or glucuronic acid. Monosaccharides also include ascorbic acid, amino acid-carrying monosaccharides and monosaccharides which carry lipid, phosphatidyl or polyol residues.

By "polynucleotide" is meant a homo- or heteropolymer of two or more nucleotide units connected by a linkage, such as a phosphodiester, phosphonate, and/or phosphorothioate linkages, among others.

The term "particles" as used herein encompasses spheres, spheroids, beads and other shapes as well. In addition, particles with the magnetic properties of superparamagnetism, ferrimagnetism and ferromagnetism are referred to herein as "magnetic particles." In the present invention, the term particles can refer to superparamagnetic starch particles, superparamagnetic, chitosan particles, or any other superparamagnetic particle with a polysaccharide matrix.

By "peptide" is meant a molecule that contains from 2 to 100 natural or unnatural amino acid residues joined by amide bonds formed between a carboxyl group of one amino acid and an amino group from the next one. The term "glycopeptide" refers to a peptide that is covalently bound to a carbohydrate.

The term "polysaccharide" is meant to include any polymer of monosaccharides, or salts therein, and includes disaccharides, oligosaccharides, etc. Polysaccharides include starch, dextran, cellulose, chitosan, glycogen, hyaluronic acid, alginic acid, and glycosylaminoglycans. The polysaccharide of this invention may be unmodified or modified and the term polysaccharide is used herein to include both types. By modified polysaccharide it is meant that the polysaccharide can be derivatized or modified by typical processes known in the art, e.g., esterification, etherification, oxidation, acid hydrolysis, cross-linking and/or enzyme conversion. Typically, modified polysaccharides include esters such as the acetate and the half-esters of dicarboxylic acids, particularly the alkenylsuccinic acids; ethers, such as hydroxyethyl and hydroxypropyl starches and starches reacted with hydrophobic cationic epoxides; starches oxidized with hypochlorite; starches reacted with cross-linking agents such as phosphorous oxychloride, epichlorohydrin or phosphate derivatives prepared by reaction with sodium or potassium orthophosphate or tripolyphosphate and combinations thereof. These and other conventional modifications of starch are described in

publications such as Starch: Chemistry and Technology, 2nd Edition, Ed. Whistler, BeMiller, and Paschall, Academic Press, 1984, Chapter X.

By "protein" is meant a molecule that contains over 100 natural or unnatural amino acid residues joined by amide bond(s) formed from a carboxyl group of one amino acid and an amino group from the next one. The term "glycoprotein" refers to a protein that is covalently bound to a carbohydrate.

By the term "superparamagnetic" is meant a material that is highly magnetically susceptible, *i.e.*, it becomes strongly magnetic when placed in a magnetic field, but like a paramagnetic material, rapidly loses its magnetism and displays no remanence once the magnetic field has been removed.

As used herein "charged group" refers to a moiety which loses a proton at physiological pH thereby becoming negatively charged (e.g., carboxylate, sulfate, phosphoramidate, borate, phosphate, phosphonate, phosphonate ester, sulfonate, sulfamate, sulfate, thiolate, phenolate, or phosphodiester), a moiety which gains a proton at physiological pH thereby becoming positively charged (e.g., ammonium, imidazolium, guanidinium, or amidinium), a moiety that includes a net formal positive charge without protonation (e.g., quaternary ammonium), or a moiety that includes a net formal negative charge without loss of a proton (e.g., borate, BR_4^-). Matrices including charged groups can be prepared, for example, by deacetylation of chitin to form chitosan (e.g., to form a primary amine), the oxidation of saccharide hydroxymethyls to carboxylates, or by chemically coupling charged groups to an available functional group of the matrix.

As used herein, the term "ammonium" refers to a charged group including a nitrogen atom, such as a primary ($-NH_3^+$), secondary ($-NRH_2^+$), tertiary ($-NR_2H^+$), or quaternary ($-NR_3^+$) nitrogen.

As used herein, the term "sulfate" refers to a charged group including a $-SO_3^-$ moiety, such as a sulfonate ($-CH_2SO_3^-$), sulfamate ($-NHSO_3^-$), or sulfate ($-OSO_3^-$) group.

Brief Description of the Drawings

FIG. 1 is a reaction scheme outlining the results from the treatment of $Fe(OH)_2$ with various oxidants.

FIG. 2 is a composite of solid-state ^{13}C-NMR spectra of Contramid® (a) and carboxylated MagCon samples (b, c).

FIG. 3 is a composite of FTIR spectra from Contramid® (a), MagCon prepared by nitrate oxidation (b), and MagCon prepared by peroxide oxidation (c).

FIG. 4 is a composite of FTIR spectra of chitosan (a), MagCon-COOH (b), MagCon-COOH conjugated with chitosan, 1:0.3 (c), and MagCon-COOH conjugated with chitosan, 1:1 (d).

FIG. 5 is a composite of FTIR spectra of MagCon conjugated to bovine serum albumin (BSA) at BSA reaction mixture concentrations of 0 mg/mL, 2 mg/mL, 10 mg/mL, and 50 mg/mL.

FIG. 6 is a composite of FTIR spectra of MagCon-COOH and MagCon-COOH conjugated to bovine serum albumin (BSA) at BSA reaction mixture concentrations of 0 mg/mL, 1.6 mg/mL, 8 mg/mL, and 40 mg/mL.

FIG. 7 is a graph depicting the effect of BSA concentration upon the binding of BSA to MagCon-NH_2 matrix.

FIG. 8 is a graph depicting the effect of BSA concentration upon the binding of BSA to MagChi matrix.

FIG. 9a is a graph depicting the effect of RNase concentration upon the binding of RNase to MagCon-30COOH matrix at pH 5.5.

FIG. 9b is a graph depicting the effect of RNase concentration upon the binding of RNase to MagCon-30COOH matrix at pH 6.0.

FIG. 10 is a graph depicting the effect of pH upon the binding of BSA to MagChi matrix.

FIG. 11 is a graph depicting the effect of pH upon the binding of RNase to MagCon-30COOH matrix.

FIG. 12 is a graph depicting the effect of IgG concentration upon the binding of IgG to MagChi-Protein A matrix graph.

Detailed Description of the Invention

The invention provides a method for the *in situ* formation of superparamagnetic particles in a cross-linked starch matrix. The method involves: (a) diffusion an Fe(II) salt into a polysaccharide matrix, thereby entrapping Fe(II) ions within the matrix and, (b) oxidizing the Fe(II) ions with nitrate under alkaline conditions, converting the polysaccharide matrix of Fe(II) ions into superparamagnetic ferric oxide particles. The formation of superparamagnetic polysaccharide matrices using mild oxidation methods reduces the oxidative degradation of the matrix during their formation. The particles can be employed as described herein for the separation of target biological materials from mixtures.

Polysaccharide Matrices

Examples of polysaccharide matrices used for the formation of superparamagnetic particles are cross-linked starch, chitosan, chitin crystallites, Sephadex™ (dextran beads cross-linked with epichlorohydrin), cellulose fibers, and Avicel® (microcrystalline cellulose), the properties of which are shown in Table 1.

Table 1. Examples of polysaccharides used for *in situ* synthesis of superparamagnetic particles.

Name	Description	Functional Group
Cross-linked starch	Epichlorohydrin cross-linked amylose, Hylon-VII, amylose content ~ 70%	-OH, can be transformed to -COOH
Chitosan	A copolymer of β-(1-4)-linked 2-amino-2-deoxy-D-glucose, readily prepared from chitin by chemical N-deacetylation	$-NH_2$, -OH
Chitin crystallites	Rod-like crystallites of chitin obtained via acid hydrolysis; size: about 1 μm	$-NH_2$, -OH
Sephadex	Epichlorohydrin cross-linked dextran beads; stable at pH 3-12; size: 20-40 μm	-OH
CLD fibres	Cellulose fibres; stable at pH 3-12	$-CH_2COOH$ and -OH
Avicel	Microcrystalline cellulose (Avicel PH-102 and PH-103); insoluble in water, dilute acids, and most organic solvents; practically insoluble in dilute NaOH solution; size: 90-180 μm	-OH, can be transformed to -COOH

Due to its favorable properties and its ready availability, cross-linked starch is a desirable polysaccharide of the present invention. The chemistry of crosslinking polysaccharides is well known and there are a variety of agents to crosslink hydroxyl groups of polysaccharides. In the present context cross-linked starch is prepared by treating granular starch with multifunctional reagents capable of forming linkages with hydroxyl groups in the starch (Park et al., "Crosslinking of water-soluble polymers" in *Biodegradable Hydrogels for Drug Delivery*, Technomic Publishing Co., USA, 1993: pgs 73-82). The two components of starch granules, amylose and amylopectin, can vary in relative amount, e.g., from about 2 percent to about 90 percent amylose. Cross-linking of amylose can be carried out in the manner described by Mateescu in *Biochimie* 60:535-37 (1978) by reacting amylose with epichlorohydrin in an alkaline medium. In the same manner, amylose can also be cross-linked with other cross-linking agents

including, but not limited to, 2,3-dibromopropanol, epichlorohydrin, sodium trimetaphosphate, linear mixed anhydrides of acetic and di- or tribasic carboxylic acids, vinyl sulfone, diepoxides, cyanuric chloride, hexahydro-1,3,5-trisacryloyltriazine, hexamethylene diisocyanate, toluene 2,4-diisocyanate, N, N-methylenebisacrylamide, N,N'-bis (hydroxymethyl)ethyleneurea, phosgene, tripolyphosphate, mixed carbonic-carboxylic acid anhydrides, imidazolides of carbonic and polybasic carboxylic acids, imidazolium salts of polybasic carboxylic acids, guanidine derivatives of polycarboxylic acids, and esters of propanoic acid. Desirably, the starch matrices utilized in the methods of the invention, the amylose content is above 40%. In a most desirable embodiment, the amylose content is above 60%. Commercially available Contramid® has an amylose content of about 70%.

Other useful polysaccharide matrices can be prepared from chitosan, a linear copolymer of β-(1-4)-linked 2-amino-2-deoxy-D-glucose, which is readily prepared from chitin by chemical N-deacetylation. In chitosan, generally about 80% of the units are deacetylated, with the remaining 20% acetylated. These values can vary with chitin sources and with processing methods. The chemical and biochemical reactivity of chitosan is higher than that of chitin because chitosan has free primary amino groups distributed regularly in its chain. Therefore, chitosan is soluble by salt formation because the primary amines can be protonated by certain selected acids (Muzarelli et al., Structural and functional versatility of chitins" in *Polysaccharides. Structural, diversity, and functional versatility*, Severian Dumitriu, Ed., Marcel Dekker, Inc. 1998, pgs. 575-77).

The choice of the polysaccharide ultimately used to form a superparamagnetic particle of the invention may depend on the functional groups (e.g., hydroxyl, amine, carboxyl) that are contained within. Superparamagnetic particles or compositions that result from the further manipulation of these functional groups include the attachment of ligands or other functional moieties. Functional group manipulation can occur either before or after Fe(II) oxidation.

Iron Oxidation

When the Fe(II) salt is brought into contact with the polysaccharide matrix, the salt is diffused in the matrix before the nitrate oxidation step. This is normally accomplished, after its addition, by mixing the mixture by any number of means, including shaking, swirling, mechanical stirring, or magnetic stirring. Most desirably, the mixture is mechanically stirred.

Examples agents suitable for providing the alkaline conditions for the first aspect of the present invention include, but are not limited to, sodium carbonate, potassium carbonate, tetraalkylammonium hydroxides, and ammonium hydroxide. Desirably, alkaline conditions are provided by contacting the matrix with ammonium hydroxide. Desirably, the pH of the reaction mixture after the addition of base is from 10 to 14. Most desirably, the pH is 12.

The source of Fe(II) can be from one or a combination of different Fe(II) salts or complexes. These sources include $Fe(OAc)_2$, $(NH_4)_2Fe(SO_4)_2$, $FeBr_2$, $FeCl_2$, FeC_2O_4 (iron oxylate), $FeSO_4$, or $Fe(ClO_4)_2$. In desirable embodiment of the first aspect of the present invention, the source of Fe(II) ions includes $FeCl_2$, $Fe(OAc)_2$, or $(NH_4)_2Fe(SO_4)_2$. Most desirably, $FeCl_2$ is used as the source of Fe(II) ions.

The oxidizing step is performed under alkaline conditions in such a manner that Fe(II) is oxidized to Fe(III). Desirably, the product of iron oxidation is the superparamagnetic species Fe_3O_4 (*i.e.*, magnetite). Oxidants that can be used for this purpose include sodium nitrate, potassium nitrate, cesium nitrate, ammonium nitrate, tetraalkylammonium nitrate, wherein alkyl is linear or branched C_1-C_8 alkyl, silver nitrate, or barium nitrate. Most desirable is when potassium nitrate is the source of nitrate.

Charged Groups

As an example of functional group manipulation, one aspect of the present invention features a method for the *in situ* oxidation of a polysaccharide matrix that includes a superparamagnetic iron particle to produce a carboxylated superparamagnetic particle or composition. An embodiment of this aspect includes superparamagnetic particles prepared by the method of the first aspect of the present invention. One method for the *in situ* oxidation of a polysaccharide matrix includes maintaining a solution or suspension of at a pH of between about 7.5 to about 10.5, desirably a pH of between about 9 to about 10, most desirably a pH of about 9.5, by the addition of a base. Exemplary bases are carbonate or hydroxide. Most desirably, the base used is ammonium hydroxide. The method also includes maintaining the basic solution/suspension of the superparamagnetic particle at a temperature of between about 0 °C and 20 °C, desirably between about 0 °C and 10 °C, most desirably between about 2 °C and 5 °C. The method includes adding sodium bromide and a catalytic amount of TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy, free radical) to the solution/suspension, followed by the addition of sodium hypochlorite. Desirably, the amount of TEMPO added is between about 2 to 7 mole percent of the mole equivalents of COOH groups desired with the amount of sodium hypochlorite used stoichiometric to the number of mole equivalents of COOH groups desired. In an embodiment of this aspect, sodium chlorite is also added to the solution suspension with the addition of sodium hypochlorite. Desirably, the sodium chlorite added is stoichiometric to, and the amount of sodium hypochlorite added is about 3 mole percent of, that of the number of mole equivalents of COOH groups desired. A procedure for the use of sodium chlorite in TEMPO-mediated oxidations can be found in Zhao et al., *J. Org. Chem.* 64:2564-6 (1999).

Affinity Ligands

Ligands having affinity for the target biological material can be either entrapped within or covalently attached to the polysaccharide matrix. For example, if the polysaccharide matrix contains a reducing sugar, a particularly useful method of attaching an affinity ligand is by a reductive amination procedure, reacting the carbonyl of the reducing sugar with an amine of the affinity ligand, followed by reduction of the resulting imine to an amine with cyanoborohydride. If the polysaccharide contains carboxylic acids, a useful method of attaching an affinity ligand is by forming amides between the polysaccharide and amines present on the affinity ligand. This can be done using standard amide bond forming reagents, desirably by the use of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDCI). If the polysaccharide contains an amine, useful ways of attaching an affinity ligand include reacting the amine on the polysaccharide with either a carbonyl or a carboxyl functionality on the affinity ligand using the conditions described above. Affinity ligands and methods of binding them to support materials are well known in the purification art, e.g., the reference texts *Affinity Separations. A Practical Approach* (Practical Approach Series), Matejtschuk (Editor), Irl Pr: 1997 and *Affinity Chromatography*, Herbert Schott, Marcel Dekker, New York: 1997.

Exemplary affinity ligands include, without limitation, antibodies or antibody fragments, antigens, biotin, steptavidin, enzyme substrates or substrate analogs, protein A or protein A analogs, and complementary polynucleotides. These affinity ligands or other biological materials can be attached to the matrix after Fe(II) oxidation or, preferably, be included in the matrix before nitrate-mediated oxidation of Fe(II).

Characterization of Superparamagnetic Polysaccharide Matrices

Several methods can be used in the analysis of the superparamagnetic particles and compositions of the invention. Iron content can be determined by

atomic absorption spectroscopy (AAS) or by extraction sample magnetometry (ESM) measurements. In ESM, about 20 mg of magnetic materials at room temperature are vibrated in a magnetic field varying from –1.5 to 1.5 T. The data of magnetization as a function of the applied field were recalculated as the percents of iron in the magnetic particles. In these calculations, the standard values of magnetization of magnetite (84 – 90 J/T kg) were employed. As shown in FIG. 1, other oxidation methods either produce non-magnetic iron species (e.g., α-FeOOH, β-FeOOH, or γ-FeOOH) or species with a reduced magnetization properties, such as γ-Fe_2O_3 or δ-FeOOH. As a result of this, in those cases where iron species other than Fe_3O_4 are produced, it is expected that iron content determined via extraction sample magnetometry be lower than iron content determined by atomic absorption spectroscopy. This is generally observed, as shown in Tables 2-4, if one compares the ESM and AAS values for the formation of superparamagnetic particles synthesized via nitrate oxidation and those prepared via peroxide oxidation. In addition to differences in the iron content values, the product yields obtained from particles via nitrate oxidation are higher. This may be due to the relative lack of polysaccharide matrix decomposition observed when nitrate is used as the oxidant.

Table 2. Characteristics of cross-linked superparamagnetic particles synthesized via peroxide oxidation (PX).

Sample[1]	Starting Material (g)	Product (g)	Yield (%)	Oxidation Cycles (No.)	Iron Content (%)[2]	
					ESM	AAS
MagConI (PX)	1.0	0.32	32.0	3	36.1	33.3
MagConII (PX)	3.5	1.62	46.3	3	10.3	28.8
MagCon 8.5COOH (PX)	2.0	0.43	21.5	3	13.8	23.3
MagCon 34COOH (PX)	3.0	0.33	11.0	3	22.0	25.3
MagChi (PX)	5.0	4.92	98.4	3	22.6	46.4

[1] I & II refer to experiment numbers, 8.5COOH and 34COOH refers to degree of carboxylation (*i.e.*, 8.5% and 34%)

[2] ESM is extraction sample magnetometry, AAS is atomic absorbance spectroscopy

Table 3. Characteristics of cross-linked superparamagnetic particles synthesized via nitrate oxidations.

Sample[1]	Starting Material (g)	Product (g)	Yield (%)	Oxidation Cycles (No.)	Iron Content (%)[2]	
					ESM	AAS
MagConI	5.0	3.91	78.2	1	6.8	6.2
MagConII	5.0	4.86	97.2	1	10.1	11.44
MagCon 30COOH	5.0	4.56	91.2	1	16.8	11.78
MagConCI	2.0	2.03	102.0	1	n/d	39.6
MagConCII	2.0	2.20	110	1	n/d	35.4
MagCon 20COOH	5.0	4.3	86.5	1	62.7	46.0

[1] I & II refer to experiment numbers, 30COOH and 20COOH refers to degree of carboxylation (*i.e.*, 30% and 20%), MagConC is derived from commercially available Contramid®.

[2] ESM is extraction sample magnetometry, AAS is atomic absorbance spectroscopy

Table 4. Characteristics of SPMPs prepared by oxidizing Fe(II) with nitrate using various polysaccharide matrices.

Sample[1]	Starting Material (g)	Product (g)	Yield (%)	Oxidation Cycles (No.)	Iron Content (%)[2]	
					ESM	AAS
MagChi	5.00	14.90	300	1	62-68	42.54
Magnetic Sephadex	2.00	1.86	93	1	14-15	9.02
MagMCC-I	5.00	1.96	98	1	8-10	6.08
MagMCC-II	3.50	5.3	106	1	n/d	5.70
Magnetic CLD fibers	3.00	0.75	100	1	31-34	24.5
Magnetic Chitin crystallites	3.00	3.1	103	1	n/d	18.07

[1] I & II refer to experiment numbers

2 ESM is extraction sample magnetometry, AAS is atomic absorbance spectroscopy

Separations

Compositions of the invention may be used for separation, isolation, identification, or purification of biological entities. For example, the composition is brought into contact with a biological material that interacts positively with an affinity ligand of the composition. Purification of the biological material is then affected by the application of a magnetic field or gradient, such as those used in high gradient magnetic separations (HGMS). The biological material can be a cell (e.g., in a method for separating cells), a virus, a phage, a protein, a peptide, a carbohydrate, a glycopeptide, a glycoprotein, a glycosylaminoglycan, a cationic lipid, a glycolipid, or a polynucleotide among others.

Magnetic assisted purification improves upon traditional purifications that rely upon filtration or centrifugation to remove cells, particles, and impurities before adsorption or chromatography. In this invention, the magnetic particles can be applied directly to a fermentation broth or crude lysate for adsorption.

To achieve efficient separations, the matrix is brought into contact with a target biological material. Optionally, the target material interacts positively with an affinity ligand incorporated into the matrix. Purification of the target material is then affected by the application of a magnetic field or gradient, such as those used in high gradient magnetic separations (HGMS). The target material can be a cell, a virus, or a phage. Typically, the target material will be a protein or a peptide which is being separated from a mixture. Once a complex has formed between the target material and the matrix, a magnetic field is then used to isolate the complex using an appropriate magnetic separator.

The methods of the invention can be used to purify biological materials, such as proteins or peptides, from bacteria, yeast, and plant or animal cell culture fluids, or extracts from tissues. The process can include clarification of a fermentation broth or an initial tissue homogenization, e.g., by removal of cells or debris by centrifugation, microfiltration, and/or adsorption to a matrix.

The materials and methods of the present invention can be employed for bulk separations using one or more established separation strategies known in the art. These include clarification and packed bed chromatography, among others.

An alternative to methods of clarification and packed bed chromatography is adsorption to the matrix in a stirred tank. This technique is useful when recovering the target biological material from a large volume of crude feed.

Bulk separation of the target biological material can also be performed in a fluidized bed containing the matrix. This technique can eliminate the need for particulate removal prior to purification using the matrix. Fluidized beds have been used in industry for many years for the recovery of antibiotics including batch-processing techniques for the recovery of streptomycin and semi-continuous systems for novobiocin. In a fluidized bed, channeling, turbulence, and backmixing is extensive, and is similar to a batch process in a stirred tank.

Alternatively, the matrix can be entrapped within a fixed apparatus, such as a filter or cartridge, designed for the mixture containing the target biological material to pass over by action of gravity or positive pressure.

The use of expanded bed adsorption (EBA) is an alternative that minimizes fouling, often associated with high protein loads (see, for example, "Expanded Bed Adsorption: Principles and Methods", Pharmacia Biotech, ISBN 91-630-5519-8). On a larger scale, the expanded bed uses an upward operating flow through the bed and the flow rate is restricted by increased viscosity, the density of the chromatographic adsorbants used, and the rate of binding of the target biological material to the adsorbant.

Other Uses

Another use of a composition of the invention is in the synthesis of polymeric biological molecules (e.g., peptides, polynucleotides). In this method, stepwise synthesis is performed using a composition of the invention as the solid support. Thus, attachment of one monomeric building block and repetitive addition of subsequent monomeric building blocks (e.g., amino acid derivatives, nucleotide derivatives) to the composition can be combined with magnetic separation at appropriate times in the synthesis for the removal of reaction by-products. Compositions of the present invention can also be used as a solid support for combinatorial chemistry.

The compositions of the invention may also be especially valuable for use as *in vivo* diagnostic agents. Particularly desirable is the use of these compositions as NMR contrast agents. Typically, the composition, in a pharmaceutically acceptable carrier, is administered to a patient, orally, intraperitoneally, or intravascularly, followed by subjecting the patient to NMR imaging.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the methods

claimed herein are performed and evaluated. They are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention.

Some of the materials were characterized using solid-state ^{13}C-NMR and/or Fourier-transform infrared spectroscopy as follows.

Solid-state ^{13}C-NMR spectra of MagCon and its carboxylated derivatives were recorded on a Chemagnetics CMX-300 spectrometer. Integral intensities of the signals at 62 ppm (C-6 hydroxymethyl) and 178 ppm (C-6 carboxyl) were used for determination of the degree of carboxylation (D.C.): D.C. (%) = [C-6 carboxyl / (C-6 hydroxymethyl + C-6 carboxyl)] x 100. Shown in FIG. 2 is a composite of solid-state ^{13}C-NMR spectra of Contramid® (a) and carboxylated MagCon samples (b, c).

Fourier-transform infrared spectra (FTIR) as an average of 100 scans with a 4 cm^{-1} resolution were recorded with a Brüker IFS 48 spectrometer. The samples (0.03 g) were prepared in the form of a pellet in KBr (0.2 g). Further evidence of differences between superparamagnetic MagCon particles obtained via nitrate oxidation and peroxide oxidation can be found by observing their FTIR spectra. FIG. 3 shows normalized spectra of Contramid®, MagCon produced by nitrate oxidation (MagCon NT), and MagCon produced by peroxide oxidation (MagCon PX). The material produced by the peroxide oxidation results in an OH-stretch band at about 3375 cm^{-1} that has greater intensity and is shifted more towards lower wavenumbers than particles obtained by nitrate oxidation. This is believed to be due to the presence of unreacted $Fe(OH)_2$ (see Ruan et al., *Spectrochim. Acta A*, 57:2575-86 (2001)) or other hydrated iron species, such as β-FeOOH, in the particles obtained via peroxide oxidation.

Example 1

In situ synthesis of superparamagnetic cross-linked starch particles (MagCon) by the nitrate mediated oxidation of Fe(II) ions

A suspension of 5 g of Contramid® (a high amylose cross-linked starch) in 100 mL of fresh deionised water was added to 250 mL of an aqueous solution of 0.5 M $FeCl_2$. The suspension was stirred under reduced pressure, thereby removing all gases from the suspension and also facilitating diffusion of Fe ions into the porous Contramid® matrix. After 30 minutes of stirring, the swollen beads of the Contramid®-Fe complex were separated by centrifugation and washed several times with deionised water. The resulting Contramid®-Fe particles were suspended in 250 mL of deionised water and 200 mL of 0.5 M NH_4OH was added, turning the mixture dark green. Immediately after NH_4OH addition, the mixture was placed into a water bath kept at 70-80 °C and 30 mL of 10% (w/w) KNO_3 was added. The reaction mixture was stirred at this temperature for 60 minutes. Nitrate oxidizes Fe(II) to Fe_3O_4 according to the following equation:

$$12\ Fe(OH)_2 + NO_3^- \rightarrow 4\ Fe_3O_4 + NH_3 + 10\ H_2O + OH^-$$

After 60 minutes, the flask was removed from the water bath and the reaction mixture was stirred for another 10 minutes. The resulting dark grey particles (MagCon particles) were collected by centrifugation, washed with water, washed with 0.1 M acetic acid, and lyophilized. The final product yield typically ranges from 3.9 g to 5.0 g, which corresponds to a recovery yield of 78 to 97%, and contains up to 50% (w/w) of iron in the form of Fe_3O_4, as determined by atomic absorbance spectroscopy (AAS) or extraction sample magnetometry (ESM), as shown in Table 3.

Example 2

***In situ* formation of the superparamagnetic chitosan particles (MagChi) by nitrate-mediated oxidation of iron (II) ions**

Chitosan (5 g) was dissolved in 100 mL of 0.1 M acetic acid to give a viscous chitosan solution. This solution was transferred into the flask containing 25 g of $FeCl_2$ in 500 mL of water and the mixture obtained was stirred under reduced pressure for 30–50 minutes. After incubation, the chitosan-Fe complex was precipitated by the addition of 200 mL of 0.5 M NH_4OH and the resultant dark-green gel was broken up by intense stirring and washing several times with deionised water. The resulting chitosan-$Fe(OH)_2$ particles were resuspended in 200 mL of deionised water and 400 mL of 0.5 M NH_4OH was added. Immediately after this, the mixture was placed into a water bath kept at 70–80 °C and 100 mL of 10% (w/w) KNO_3 in water was added. The reaction mixture was stirred at this temperature for 60 to 90 minutes. After this time, the flask was removed from the water bath and the reaction mixture stirred for another 10 minutes. The resultant dark grey or black particles were collected by centrifugation, washed with deionised water, and lyophilized. The final product yield typically contains up to 70% (w/w) of iron in the form of Fe_3O_4, as determined by AAS or ESM, as shown in Table 4. The content of iron, as well as the recovery yield of the particles produced, can be regulated by the $FeCl_2$ concentration used in the formation of the chitosan-Fe(II) complex.

Example 3

***In situ* synthesis of superparamagnetic Sephadex particles by the nitrate mediated oxidation of Fe(II) ions**

A suspension of 5 g of Sephadex™ (epichlorohydrin cross-linked dextran beads, 20–40 μm in size) in 100 mL of fresh deionised water was added to 250 mL of an aqueous solution of 0.5 M $FeCl_2$. The suspension was stirred under reduced pressure, thereby removing all gases from the suspension and also facilitating

diffusion of Fe ions into the porous Sephadex matrix. After 30 minutes of stirring, the swollen beads of the Sephadex-Fe complex were separated by centrifugation and washed several times with deionised water. The resulting Sephadex-Fe particles were suspended in 250 mL of deionised water and 200 mL of 0.5 M NH_4OH was added. Immediately after NH_4OH addition, the mixture was placed into a water bath kept at 70-80 °C and 30 mL of 10% (w/w) KNO_3 was added. The reaction mixture was stirred at this temperature for 80 minutes. After this time, the flask was removed from the water bath and the reaction mixture was stirred for another 10 minutes. The resulting particles were collected by centrifugation, washed with deionised water, washed with 0.1 M acetic acid, and lyophilized. The final product yield typically contains up to 15% (w/w) of iron in the form of Fe_3O_4, as determined by AAS or ESM, as shown in Table 4.

Example 4

***In situ* synthesis of superparamagnetic cellulose fiber particles by the nitrate mediated oxidation of Fe(II) ions**

Two pieces of cellulose sheets were weighed to give 0.75 g of the material (CLD fibers). The material was placed in 100 mL of deionised water to swell (15 minutes) and the hydrogel thus obtained transferred to a 10% (w/w) $FeCl_2$ solution in water (150 mL) to form a yellow mixture. After 40 minutes of stirring under vacuum, the cellulose-Fe complex were separated by centrifugation and washed several times with deionised water. The resulting Fe-cellulose fiber particles were suspended in 100 mL of deionised water and 200 mL of 0.5 M NH_4OH containing 1.0 g of KNO_3 was added. The reaction mixture was stirred at 70 °C for 40 minutes. The resulting magnetic fibers were collected by centrifugation, washed with deionised water, washed with acetone, and dried *in vacuo*. The final product yield typically contains up to 35% (w/w) of iron in the form of Fe_3O_4, as determined by ESM, as shown in Table 4.

Example 5

In situ synthesis of superparamagnetic chitin crystallites by the nitrate mediated oxidation of Fe(II) ions

Chitin (10 g) was treated with a 5% (w/w) solution of sodium hypochlorite (250 mL) in water at 50 °C for one hour. The suspension was filtered and the oxidized chitin subsequently hydrolyzed in 100 mL of boiling 2.5 M HCl for 1 hour. The chitin suspension was then subjected to washing via centrifugation-dilution cycles with deionised water. When the pH approached 2.0–2.6, the material formed a colloidal suspension that could not be further separated from solution by slow-speed centrifugation. This suspension was then placed into dialysis tubes with a molecular weight cutoff limit of about 13-15 kDa and dialyzed against distilled water until a pH of 5.5-6.0 was achieved for the suspension.

The resulting suspension was added to 250 mL of an aqueous solution of 0.5 M $FeCl_2$. The suspension was stirred under reduced pressure, thereby removing all gases from the suspension and also facilitating diffusion of Fe ions into the chitin matrix. After 30 minutes of stirring, chitin-Fe complex was separated by centrifugation and washed several times with deionised water. The resulting chitin-Fe particles were suspended in 250 mL of deionised water and 200 mL of 0.5 M NH_4OH was added. Immediately after NH_4OH addition, the mixture was placed into a water bath kept at 70-80 °C and 30 mL of 10% (w/w) KNO_3 was added. The reaction mixture was stirred at this temperature for 60 minutes. After this time, the flask was removed from the water bath and the reaction mixture was stirred for another 10 minutes. The resulting particles were collected by centrifugation, washed with deionised water, washed with 0.1 M acetic acid, and lyophilized.

Example 6

In situ synthesis of superparamagnetic microcrystalline cellulose (Avicel®) particles by the nitrate mediated oxidation of Fe(II) ions

A suspension of 5 g of Avicel® (microcystalline cellulose) in 100 mL of fresh deionised water was added to 250 mL of an aqueous solution of 0.5 M $FeCl_2$. The suspension was stirred under reduced pressure, thereby removing all gases from the suspension and also facilitating diffusion of Fe ions into the Avicel® matrix. After 30 minutes of stirring, the Avicel®-Fe complex was separated by centrifugation and washed several times with deionised water. The resulting Avicel®-Fe particles were suspended in 250 mL of deionised water and 200 mL of 0.5 M NH_4OH was added. Immediately after NH_4OH addition, the mixture was placed into a water bath kept at 70-80 °C and 30 mL of 10% (w/w) KNO_3 was added. The reaction mixture was stirred at this temperature for 60 minutes. After this time, the flask was removed from the water bath and the reaction mixture was stirred for another 10 minutes. The resulting particles, MagMCC, were collected by centrifugation, washed with deionised water, washed with 0.1 M acetic acid, and lyophilized. The final product yield typically contains up to 10% (w/w) of iron in the form of Fe_3O_4, as determined by absorbance spectrometry (see Table 4).

Example 7

TEMPO-mediated oxidation of MagCon particles to form MagCon-COOH

A suspension of 5 g of superparamagnetic cross-linked starch particles (MagCon particles) in 200 mL of deionised water was cooled to 2 °C. TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl, 0.02 g) and NaBr (0.4g) were dissolved in 50 mL, of deionised water and then mixed with the MagCon suspension. The pH of this suspension was adjusted to 9.5 with 0.5M NaOH. A solution of sodium hypochlorite (60 mL, available chlorine 10-13%), also cooled to 2 °C and with the pH adjusted to 9.5 with 0.5M NaOH, was added to the MagCon suspension,

keeping the reaction temperature at 2–5 °C. The reaction mixture was stirred at 2–5 °C, while keeping the pH at 9.5 with 0.5M NaOH. After 30 minutes, the reaction was stopped by the addition of several drops of ethanol and the addition of 3M aqueous HCl to a pH of 7.0 or less. The resulting MagCon-COOH particles were separated by centrifugation, washed with deionised water several times, and lyophilized. Solid-state ^{13}C-NMR spectra of MagCon and its carboxylated derivatives were recorded on a Chemganetics CMX-300 spectrometer. Integral intensities of the signals at 62 ppm (C-6 hydroxymethylene) and 178 ppm (C-6 carboxyl) (see FIG. 2) were used for determination of the degree of carboxylation (D.C.): D.C. (%) = [C-6 carboxyl / (C-6 hydroxymethylene + C-6 carboxyl)] x 100. The degree of oxidation was determined to be in the range of 5–35%.

Example 8

Synthesis of amino-containing of MagCon particles

A suspension was formed with MagCon-COOH particles (1.0 g) in a 50 mL solution of chitosan (0.3 g to 1.0 g) in 50 mL of 0.1 M acetic acid. The mixture was rapidly stirred to produce a fine suspension. The pH of the suspension was raised to 10 with 0.1 M NaOH and the precipitated MagCon-COOH-chitosan salt was magnetically separated from the solution. The particles were washed with 0.05 M KH_2PO_4 buffer (pH, 5.0) and re-suspended in 50 mL of distilled water. To the stirred suspension was added EDC (1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide, 0.25 g) and stirring was continued for 3 hours. The resulting MagCon-Chitosan particles were separated magnetically, washed with deionised water, and lyophilized. FTIR spectra of chitosan (a), MagCon-COOH (b), MagCon-COOH conjugated with chitosan, 1:0.3 (c), and MagCon-COOH conjugated with chitosan, 1:1 (d) are shown in FIG. 4.

Example 9

Attachment of bovine serum albumin (BSA) to MagCon particles

Following is a general procedure that can be used to couple proteins, or any other biological molecule containing free primary amines, to MagCon particles that have been formed via the nitrate oxidation of Fe(II).

A 0.01 M phosphate buffer in 0.15 M NaCl was prepared by dissolving KH_2PO_4 (1.74 g) and NaCl (8.7 g) in 800 mL of water and adjusting the pH of this buffer solution to 6.8–7.2 with phosphoric acid, followed by adjusting the volume of the solution to 1.0 L. MagCon particles (25 mg) were suspended in 5 mL of the phosphate buffer and the suspension was mixed for 30 minutes to allow the particles to swell. The particles were separated magnetically and the supernatant was removed. The particles were suspended in and the separated from phosphate buffer three additional times. To the particles were added 5 mL of a 50 mM sodium periodate solution (1.08 g of $NaIO_4$ in 100 mL of H_2O) and the suspension was shaken well and incubated for 30 minutes at room temperature. The particles were separated magnetically and the supernatant removed to produce activated MagCon particles. The particles were then suspended in and magnetically separated from phosphate buffer three times. A solution of bovine serum albumin (BSA) was prepared by dissolving 5–50 mg of the protein in 1 mL of phosphate buffer and this solution was transferred to a tube containing the activated MagCon particles from above. The tube was shaken and incubated for 2–3 hours at room temperature. A fixation solution containing 1 g of sodium cyanoborohydride in 100 mL of deionised water was prepared and 0.25 mL was immediately added to the mixture of MagCon particles reacted with protein. The tube was well shaken for 30 minutes, followed by magnetically separating the particles and removing the unreacted protein solution. A quenching solution was prepared by dissolving 7.5 g of glycine in 90 mL of deionised water, adjusting the pH to 8.0 with 1.0 M NaOH, and adjusting the volume of the solution to 100 mL with deionised water. To the MagCon-protein particles formed above was added 5 mL of the glycine

quenching solution and 0.5 mL of the cyanoborohydride fixation solution. The suspension was mixed well for 1 hour and the particles magnetically separated. A wash buffer was prepared by dissolving 1.21 g of Tris buffer, 1.0 g of sodium azide, 8.7 g of NaCl, and 0.37 g of EDTA in 800 mL of deionised water. The pH of the wash buffer was adjusted to 7.0 – 7.2 with 0.1 M HCl and the volume adjusted to 1.0 L. The magnetic particles were treated with the wash buffer four times. Each time the particles were well shaken with the buffer followed by magnetic separation and removal of the buffer. After this wash sequence, the protein-bound MagCon particles were ready for use. FIG. 5 is a composite of FTIR spectra of MagCon conjugated to bovine serum albumin (BSA) at BSA reaction mixture concentrations of 0 mg/mL, 2 mg/mL, 10 mg/mL, and 50 mg/mL. Amide N-H and C-N vibrations were observed in the FTIR of the protein-conjugated beads, as shown in FIG. 5.

Example 10

Attachment of Bovine Serum Albumin (BSA) to MagChi

Following is a general procedure that can be used to crosslink proteins, or any other biological molecule containing free primary amines, to MagChi particles, or any other superparamagnetic particles containing a primary amine, which have been formed via the nitrate oxidation of Fe(II).

A 0.01 M phosphate buffer in 0.15 M NaCl was prepared by dissolving KH_2PO_4 (1.74 g) and NaCl (8.7 g) in 800 mL of deionised water and adjusting the pH of this buffer solution to 6.8–7.2 with phosphoric acid, followed by adjusting the volume of the solution to 1.0 L. MagChi particles (25 mg) were suspended in 5 mL of the phosphate buffer and the suspension was mixed for 30 minutes to allow the particles to swell. The particles were separated magnetically and the supernatant was removed. The particles were suspended in and the separated from phosphate buffer three additional times. To the particles were added 5 mL of a 5% (v/v) solution of glutaraldehyde in the phosphate buffer prepared above and the

suspension was shaken well and incubated for 30 minutes at room temperature. The particles were separated magnetically and the supernatant removed to produce activated MagChi particles. The particles were then suspended in and magnetically separated from phosphate buffer three times. A solution of bovine serum albumin (BSA) was prepared by dissolving 5–50 mg of the protein in 1 mL of phosphate buffer and this solution was transferred to a tube containing the activated MagChi particles from above. The tube was shaken and incubated for 3–5 hours at room temperature. A fixation solution containing 1 g of sodium cyanoborohydride in 100 mL of deionised water was prepared and 0.25 mL was immediately added to the mixture of MagChi particles that had been treated with protein. The tube was well shaken for 30 minutes, followed by magnetically separating the particles and removing the unreacted protein solution. A quenching solution was prepared by dissolving 7.5 g of glycine in 90 mL of deionised water, adjusting the pH to 8.0 with 1.0 M NaOH, and adjusting the volume of the solution to 100 mL with deionised water. To the MagChi-protein particles formed above was added 5 mL of the glycine quenching solution and 0.5 mL of the cyanoborohydride fixation solution. The suspension was mixed well for 1 hour and the particles magnetically separated. A wash buffer was prepared by dissolving 1.21 g of Tris buffer, 1.0 g of sodium azide, 8.7 g of NaCl, and 0.37 g of EDTA in 800 mL of deionised water. The pH of the wash buffer was adjusted to 7.0–7.2 with 0.1 M HCl and the volume adjusted to 1.0 L. The magnetic particles were treated with the wash buffer four times. Each time the particles were well shaken with the buffer followed by magnetic separation and removal of the buffer. After this wash sequence, the protein-bound MagChi particles were ready for use. A comparison of the amount of protein conjugated to MagCon and MagChi particles is shown in Table 5.

Table 5. Amount of bovine serum albumin (BSA) conjugated to MagCon and MagChi particles, as determined by mass balance.

Reaction concentration of BSA (mg/mL)	Ratio of BSA conjugated to MagChi (mg of protein/mg of particle)	Ratio of BSA conjugated to MagCon (mg of protein/mg of particle)
1.0	0.000	0.002
2.0	0.002	0.023
3.0	0.010	0.033
5.0	0.027	0.055
7.5	0.048	0.082
10	0.057	0.110
25	0.093	0.176
50	0.013	0.221

Example 11

Attachment of bovine serum albumin (BSA) to MagCon-COOH

Following is a general procedure that can be used to crosslink proteins, or any other biological molecule containing free primary amines, to MagCon-COOH particles (2.0% COOH, 5.0% COOH, 10.0% COOH, 25.0% COOH, 50% COOH were used) or any other superparamagnetic particles containing a carboxyl group, which have been formed via the nitrate oxidation of Fe(II).

A 0.01 M phosphate buffer in 0.15 M NaCl was prepared by dissolving KH_2PO_4 (1.74 g) and NaCl (8.7 g) in 800 mL of deionised water and adjusting the pH of this buffer solution to 6.8–7.2 with phosphoric acid, followed by adjusting the volume of the solution to 1.0 L. MagCon-COOH particles (25 mg) were suspended in 5 mL of the phosphate buffer and the suspension was mixed for 30 minutes to allow the particles to swell. The particles were separated magnetically and the supernatant was removed. The particles were suspended in and the separated from phosphate buffer three additional times. A solution of bovine serum albumin (BSA) was prepared by dissolving 5–50 mg of the protein in 1 mL of phosphate buffer and this solution was transferred to a tube containing the

MagCon-COOH particles from above. To the particles were added 0.25 mL of a 0.2% (w/w) solution of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDCI) in water. The suspension was shaken well and incubated for 2–3 hours at room temperature. The particles were separated magnetically and the supernatant removed to produce MagCon-COOH protein particles. A quenching solution was prepared by dissolving 7.5 g of glycine in 90 mL of deionised water, adjusting the pH to 8.0 with 1.0 M NaOH, and adjusting the volume of the solution to 100 mL with deionised water. To the MagCon-COOH-protein particles formed above was added 5 mL of the glycine quenching solution. The suspension was mixed well for 1 hour and the particles magnetically separated. A wash buffer was prepared by dissolving 1.21 g of Tris buffer, 1.0 g of sodium azide, 8.7 g of NaCl, and 0.37 g of EDTA in 800 mL of deionised water. The pH of the wash buffer was adjusted to 7.0–7.2 with 0.1 M HCl and the volume adjusted to 1.0 L. The magnetic particles were treated with the wash buffer four times. Each time the particles were well shaken with the buffer followed by magnetic separation and removal of the buffer. After this wash sequence, the protein-bound MagCon-COOH particles were ready for use. A comparison of the ratio of BSA incorporation to MagCon-COOH particles of differing carboxylation percentages is shown in Table 6. Amide N-H and C-N vibrations were observed in the FTIR of the protein-conjugated beads, as shown in FIG. 6.

Table 6. Amount of bovine serum albumin (BSA) conjugated to MagCon-COOH, as determined by mass balance.

Reaction concentration of BSA (mg/mL)	Ratio of BSA conjugated to MagCon-8.5% COOH (mg of protein/mg of particle)	Ratio of BSA conjugated to MagCon-12% COOH (mg of protein/mg of particle)	Ratio of BSA conjugated to MagCon-30% COOH (mg of protein/mg of particle)
1.6	0.0002	0.0021	n/d
4.0	0.215	0.051	0.054
8.0	0.051	0.085	0.149
20.0	0.099	0.132	0.235
40.0	0.131	0.166	0.371

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Example 12

TEMPO-mediated oxidation of microcrystalline cellulose (Avicel®)

Microcrystalline cellulose (5 g, Avicel®) was suspended in 200 mL of deionised water. TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl, 0.02 g) and NaBr 10 (0.4g) were dissolved in 50 mL, of deionised water and then mixed with the MCC suspension. The pH of this suspension was adjusted to 10-11 with 0.5M NaOH. A solution of sodium hypochlorite (60 mL, available chlorine 13%), also with the pH adjusted to 10-11 with 0.5M NaOH, was added to the MCC suspension. The reaction mixture was stirred for 4 hours, followed by quenching with the addition 15 of ethanol (4 mL) and 5M HCl (5 mL). The oxidized MCC crystallites were separated from the suspension by filtration and were washed several times with ethanol. Titration and solid-state ^{13}C-NMR, see Example 7, indicate that the degree of carboxylation is about 12%.

Example 13

In situ synthesis of superparamagnetic MCC-COOH particles by the nitrate mediated oxidation of Fe(II) ions

To a suspension of 5 g of MCC-COOH in 100 mL of fresh deionised water was added aqueous ammonium ferrous sulfate (10% w/w solution, 500 mL) dropwise. The suspension was stirred for 1 hour under reduced pressure, thereby removing all gases from the suspension and also facilitating diffusion of Fe ions into the MCC-COOH matrix. The MCC-COOH-Fe complex was separated by centrifugation and washed several times with deionised water. The resulting particles were re-suspended in 100 mL of deionised water and 200 mL of 0.5 M NH_4OH was added. Immediately after NH_4OH addition, the mixture was placed into a water bath kept at 70-80 °C and 30 mL of 10% (w/w) KNO_3 was added. The reaction mixture was stirred at this temperature for 60 minutes. After this time, the flask was removed from the water bath and the reaction mixture was stirred for another 10 minutes. The resulting particles were collected by centrifugation, washed with deionised water, washed with 0.1 M acetic acid, and lyophilized. The final product contained 4.79% (w/w) of iron in the form of Fe_3O_4, as determined by the saturation magnetization of the particles.

Example 14

TEMPO-mediated oxidation of MagMCC to form MagMCC-COOH

Superparamagnetic microcrystalline cellulose was prepared starting from Avicel® (5 g) and using the method described in Example 13. The particles were oxidized using TEMPO as described in Example 12. Titration and solid-state ^{13}C-NMR, see Example 7, indicate that the degree of carboxylation is again about 12%. The final product yield contained 3.82% (w/w) of iron in the form of Fe_3O_4, as determined by the saturation magnetization of the particles.

Example 15

Attachment of bovine serum albumin (BSA) to superparamagnetic microcrystalline cellulose materials.

BSA was conjugated to the products of Examples 6, 13, and 14, using the method of Example 11. The percentage of the BSA attached was determined by protein analysis of the unbound fraction remaining in the supernatent following conjugation. For the product of Example 6, MagMCC, 8.0% of the BSA remained bound. For the product of Example 13, MagMCC-COOH prepared by cellulose oxidation followed by magnetization, 17.4% of the BSA remained bound. For the product of Example 14, MagMCC-COOH prepared by magnetization followed by cellulose oxidation, 21.5% of the BSA remained bound.

Example 16

Preparation of MagChi-NMe_3

Glycidyl trimethylammonium chloride (4 mL) was added to a suspension of chitosan (80% deacetylated, 1 g) in distilled water (25 mL). The reaction mixture was allowed to stir for 3 hours under a nitrogen atmosphere at 70 °C. The resulting chitosan-NMe_3 material was then centrifuged and washed repeatedly with distilled water and acetone and dried under ambient conditions.

Ammonium ferrous sulfate (15 g in 100 mL) was added dropwise to 1 g of chitosan-NMe_3 dissolved in 0.1N acetic acid (40 mL) to yield a yellow solution. After stirring under vacuum for 90 minutes, the solution was added to 50 mL of 0.5N NH_4OH. The resulting dark green suspension was centrifuged and washed several times with distilled water. The particles were resuspended in a mixture of water (70 mL) and 0.5N NH_4OH (80 mL). The mixture was placed into a water bath kept at 70-80 °C and 30 mL of 10% (w/w) KNO_3 was added. The reaction mixture was stirred at this temperature for 60 minutes. The resulting black suspension was washed several times with distilled water and acetone and dried under ambient conditions.

An infrared spectrum of the modified chitosan contains an additional signal attributable to the trimethylammonium group at 1479 cm^{-1}. The final product contained 20.76% (w/w) of iron in the form of Fe_3O_4, as determined by the saturation magnetization of the particles.

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Example 17

Preparation of MagChi-N-Sulfate

Sulfur trioxide trimethylamine complex (4 g) was added to a suspension of chitosan (80% deacetylated, 1 g) in distilled water (200 mL). The reaction
10 mixture was allowed to stir for 4 hours 60-70 °C. The resulting gel, chitosan-N-sulfate, was centrifuged and washed two times with water and two times with acetone and dried under ambient conditions.

Ammonium ferrous sulfate (7.5 g in 100 mL) was added dropwise to 0.5 g of chitosan-N-sulfate dissolved in 0.1N acetic acid (20 mL) to yield a cloudy white
15 solution. After stirring under vacuum for 90 minutes, the solution was added to 25 mL of 0.5N NH_4OH. The resulting dark green suspension was centrifuged and washed several times with distilled water. The particles were resuspended in a mixture of water (40 mL) and 0.5N NH_4OH (40 mL). The mixture was placed into a water bath kept at 70-80 °C and 10 mL of 10% (w/w) KNO_3 was added.
20 The reaction mixture was stirred at this temperature for 60 minutes. The resulting black suspension was washed several times with distilled water and acetone and dried under ambient conditions. The final product contained 4.79% (w/w) of iron in the form of Fe_3O_4, as determined by the saturation magnetization of the particles.

25 An infrared spectrum of the modified chitosan contains additional signals attributable to the sulfate group between 1150cm^{-1} and 1250 cm^{-1}. The final product contained 17.73% (w/w) of iron in the form of Fe_3O_4, as determined by the saturation magnetization of the particles.

Example 18

Effect of BSA Concentration on BSA Binding to MagCon-NH_2.

Five milligrams of MagCon-NH_2 matrix were equilibrated with 50 mM sodium acetate pH 5.5 for 10 minutes in a 1.5 mL test tube. The matrix was separated from the supernatant by allowing the matrix to settle in the presence of a magnetic field. Bovine Serum Albumin (Sigma Cat. No. A-7638) (BSA) samples were loaded onto the matrix and washed with the same buffers. BSA was eluted with 1 M NaCl in 50 mM Tris pH 10.0.

The amount of BSA bound and eluted was monitored using a reverse phase YMC ODS-AQ 3mm, 100x4.6 mm HPLC column on a ThermoSeparations HPLC system. The mobile phase used was water/acetonitrile/0.1% TFA. The column temperature was maintained at 30 °C. Estimations of the product concentration and purity were based on peak area integration at 280 nm using PC1000 software.

The effect of BSA concentration on the binding of BSA at pH 5.5 can be seen in FIG. 7. Saturation binding was not observed under the concentrations tested. At each concentration tested, nonspecific binding occurred that could not be eluted.

Example 19

Effect of BSA Concentration on BSA Binding to MagChi.

Five milligrams of MagChi matrix were equilibrated with 50 mM sodium acetate pH 5.5 for 10 minutes in a 1.5 mL test tube. The matrix was separated from the supernatant by allowing the matrix to settle in the presence of a magnetic field. Bovine Serum Albumin (Sigma Cat. No. A-7638) (BSA) samples were loaded onto the matrix and washed with the same buffers. BSA was eluted with 1 M NaCl in 50 mM Tris pH 10.0.

The amount of BSA bound and eluted was monitored using a reverse phase chromatography as described in Example 18.

The effect of BSA concentration on the binding of BSA at pH 5.5 can be seen in FIG. 8. Saturation binding was not observed under the concentrations tested. At each concentration tested, nonspecific binding occurred that could not be eluted.

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Example 20

Effect of RNase Concentration on RNase Binding to MagCon-30COOH.

Five milligrams of MagCon-30COOH matrix were equilibrated with 50 mM sodium acetate pH 5.5 for 10 minutes in a 1.5 mL test tube. The matrix was 10 separated from the supernatant by allowing the matrix to settle in the presence of a magnetic field. Ribonuclease A (Sigma Cat. No. R-6513) (RNase) samples were loaded and washed with the same buffers. RNase was eluted with 1 M NaCl in 50 mM Tris pH 10.0.

The amount of RNAse bound and eluted was monitored using a reverse 15 phase chromatography as described in Example 18.

The experiment was repeated at pH 6.0 and the amount of RNAse bound and eluted was monitored using a reverse phase chromatography as described in Example 21.

The effect of RNase concentration on the binding of RNAase at pH 5.5 and 20 pH 6.0 can be seen in FIGs. 9a and 9b, respectively. Saturation binding was observed at an RNase concentration between1 and 2 mg/ml. Nonspecific binding was not observed.

Example 21

25 **Effect of pH on BSA Binding to MagChi.**

Five milligrams of MagChi matrix were equilibrated with 50 mM sodium acetate, pH 5.1 to 6.0, for 10 minutes in a 1.5 mL test tube. The matrix was separated from the supernatant by allowing the matrix to settle in the presence of a magnetic field. Bovine Serum Albumin (Sigma Cat. No. A-7638) (BSA) samples

were loaded onto the matrix and washed with the same buffers. BSA was eluted with 1 M NaCl in 50 mM Tris pH 10.0.

The amount of BSA bound and eluted was monitored using a Hamilton PRP-Infinity 4mm, 30x4.1 mm column on a ThermoSeparations HPLC system. The mobile phase used was water/acetonitrile/0.1% TFA. The column temperature was maintained at 30 °C.

The effect of pH on the binding of BSA at a concentration of 0. 5 mg/ml can be seen in FIG. 10. At the pH's tested pH 5.5 was the optimum. Binding would be expected to be less outside of the pH range (5.1 to 6.0) tested because of the charge change outside of the isoelectric point of the protein and matrix.

Example 22

Effect of pH on RNase Binding to MagCon-30COOH.

Five milligrams of MagCon-30COOH matrix were equilibrated with 50 mM sodium acetate, pH 4.0 to 6.0 or Tris-HCl 7.0 to 8.0, for 10 minutes in a 1.5 mL test tube. The matrix was separated from the supernatant by allowing the matrix to settle in the presence of a magnetic field. Ribonuclease A (Sigma Cat. No. R-6513) (RNase) samples were loaded and washed with the same buffers. RNase was eluted with 1 M NaCl in 50 mM Tris pH 10.0.

The amount of RNase bound and eluted was monitored using reverse phase chromatography as described in Example 21.

The effect of pH on the binding of RNase at a concentration of 0.5 mg/ml can be seen in FIG. 11. Binding would be expected to be less outside of the pH range (4.0 - 8.0) tested because of the charge change outside of the isoelectric point of the protein and matrix.

Binding kinetics were evaluated at an RNase concentration of 0.5 mg/ml. Samples were incubated for 5, 10, and 15 minutes. Only the 5 minute sample showed incomplete binding at a level of less than 0.1 %. The other samples bound all of the RNase.

Example 23

Preparation of MagChi-Protein A Matrix.

MagChi particles (25 mg) were suspended in 5 mL of phosphate buffer saline (PBS) following three rinsings in this same buffer (0.01 M phosphate buffer, 0.15 M NaCl, pH 7.0 ± 0.2). Glutaraldehyde (5 mL of a 5% solution in PBS) were added to the particles and incubated at room temperature for three hours. The particles were separated from the solution magnetically and rinsed in PBS three times. Between 5 and 50 mg of Protein A were dissolved in 1 mL PBS and added to the glutaraldehyde-treated MagChi particles. The solution was shaken and incubated at room temperature for three to five hours. A solution of freshly prepared sodium cyanoborohydride solution (0.25 mL, 0.01 g/mL in water) was added to the reaction mixture and incubated overnight. The particles were then magnetically separated from unreacted protein remaining in the supernatent. Glycine (5 mL, 1M solution in water, pH adjusted to 8.0 with NaOH) was added to the particles along with another 0.5 mL of freshly prepared sodium cyanoborohydride solution. The solution was allowed to sit for one hour before the particles were magnetically separated from the supernatent. The particles were rinsed three times in wash buffer (1.21 g Tris, 1.0 g NaN_3, 8.7 g NaCl, 0.37 g EDTA, pH adjusted to 7.1 ± 0.1 with HCl, in approximately 1 L of water). The resulting MagChi matrix is modified by covalent linkage to protein A (4.6 mg protein A/gram of matrix).

Example 24

Effect of IgG Concentration on IgG Binding to MagChi-Protein A.

Ten milligrams of MagChi-Protein A matrix were equilibrated with 50 mM sodium acetate at pH 7.0 for 10 minutes in a 1.5 mL test tube. The matrix was separated from the supernatant by allowing the matrix to settle in the presence of a magnetic field. Human IgG (Sigma Cat. No. I-4506) (IgG) samples were loaded

and washed with the same buffer. IgG was eluted with 100 mM sodium acetate at pH 3.5.

The amount of IgG bound and eluted was monitored using reverse phase chromatography as described in Example 21.

The binding curve can be seen in FIG. 12. Under the conditions tested the MagChi-Protein A matrix showed saturation binding at a level of 2.5 mg of IgG protein per mg of matrix. Generally, greater than 90% of the IgG bound could be recovered.

Other Embodiments

All publications, patent applications, and patents mentioned in this specification are incorporated herein by reference.

While the invention has been described in connection with specific embodiments, it will be understood that it is capable of further modifications. Therefore, this application is intended to cover any variations, uses, or adaptations of the invention that follow, in general, the principles of the invention, including departures from the present disclosure that come within known or customary practice within the art.

Other embodiments are within the claims. What we claim is: